PB89-103188

Methodology for Assessing Respiration and Cellular Incorporation of Radiolabeled Substrates by Soil Microbial Communities (Journal Version)

North Carolina Univ., Chapel Hill

Prepared for

Robert S. Kerr Environmental Research Lab.
Ada, OK

1988

U.S. Department of Commerce
National Technical Information Service
NTIS

TECHNICAL REPORT DATA
(Please read Instructions on the reverse before completing)

1. REPORT NO.	2.	3. REC PB89-103188
EPA/600/J-88/065		

4. TITLE AND SUBTITLE	5. REPORT DATE
METHODOLOGY FOR ASSESSING RESPIRATION AND CELLULAR INCORPORATION OF RADIOLABELED SUBSTRATES BY SOIL MICROBIAL COMMUNITIES (Journal Version)	1988
	6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S)	8. PERFORMING ORGANIZATION REPORT NO.
Durell C. Dobbins and Frederick K. Pfaender	
9. PERFORMING ORGANIZATION NAME AND ADDRESS	10. PROGRAM ELEMENT NO.
Department of Environmental Sciences & Engineering School of Public Health University of North Carolina Chapel Hill, North Carolina 27514	CBPC1A
	11. CONTRACT/GRANT NO.
	CR-811828
12. SPONSORING AGENCY NAME AND ADDRESS	13. TYPE OF REPORT AND PERIOD COVERED
Robert S. Kerr Environmental Research Lab. - Ada, OK U.S. Environmental Protection Agency Post Office Box 1198 Ada, OK 74820	Journal Article
	14. SPONSORING AGENCY CODE
	EPA/600/15

15. SUPPLEMENTARY NOTES

Published: MICROB ECOL (1988) 15(3):257-273, May 1988.

16. ABSTRACT

A method is described for determining biodegradation kinetics of both naturally occurring and xenobiotic compounds in surface and subsurface soil samples. The method measures both respiration and uptake into cellular biomass of ^{14}C-labeled substrates. The estimation of biomass incorporation entailed removal of cells from soil particles by washing the soil with a polyvinyl-pyrrolidone/pyrophosphate solution and H_2O_2. After separation of the cells and the soil particles by centrifugation, the cells were trapped on membrane filters for liquid scintillation counting. Mass balances were easily obtained. The technique was used to measure metabolic activity in soil profiles, including unsaturated and saturated zones. First order rate constants (K_1) were in the range of 10^{-3}-10^{-2} hour^{-1} for amino acid metabolism and 10^{-5}-10^{-4} hour^{-1} for m-cresol metabolism. Saturation kinetics were observed for amino acids and m-cresol. m-Cresol K_1 values for uptake often exceeded those for respiration by greater than a factor of ten. V_{max} values were low (amino acids, 10^1-10^2ng g^{-1} hour^{-1}; m-cresol, 10^{-1}ng g^{-1} hour^{-1}), whereas K_m values were quite high (amino acids, 10^3-10^4ng g^{-1}; m-cresol 10^3-10^5ng g^{-1}). Saturation was not observed in many horizons even at 10^5ng g^{-1} dry soil. Frequently, respiration obeyed saturation kinetics whereas uptake was first order. It is concluded that measuring only kinetics of respiration may lead to severe underestimations of biodegradation rates.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group

REPRODUCED BY
U.S. DEPARTMENT OF COMMERCE
NATIONAL TECHNICAL
INFORMATION SERVICE
SPRINGFIELD, VA 22161

18. DISTRIBUTION STATEMENT	19. SECURITY CLASS (This Report)	21. NO. OF PAGES
RELEASE TO PUBLIC	UNCLASSIFIED	19
	20. SECURITY CLASS (This page)	22. PRICE MF PC $12.95/$6.95
	UNCLASSIFIED	

EPA Form 2220-1 (Rev. 4-77) PREVIOUS EDITION IS OBSOLETE

EPA/600/J-88/065
JOURNAL ARTICLE

Microb Ecol (1988) 15:257–273

MICROBIAL ECOLOGY
© Springer-Verlag New York Inc. 1988

NTIS is authorized to reproduce and sell this report. Permission for further reproduction must be obtained from the copyright owner.

Methodology for Assessing Respiration and Cellular Incorporation of Radiolabeled Substrates by Soil Microbial Communities

Durell C. Dobbins and Frederic K. Pfaender

Department of Environmental Sciences and Engineering, School of Public Health, University of North Carolina, Chapel Hill, North Carolina 27514 USA

Abstract. A method is described for determining biodegradation kinetics of both naturally occurring and xenobiotic compounds in surface and subsurface soil samples. The method measures both respiration and uptake into cellular biomass of ^{14}C-labeled substrates. The estimation of biomass incorporation entailed removal of cells from soil particles by washing the soil with a polyvinyl-pyrrolidone/pyrophosphate solution and H_2O_2. After separation of the cells and the soil particles by centrifugation, the cells were trapped on membrane filters for liquid scintillation counting. Mass balances were easily obtained. The technique was used to measure metabolic activity in soil profiles, including unsaturated and saturated zones. First order rate constants (K_1) were in the range of 10^{-3}–10^{-2} hour^{-1} for amino acid metabolism and 10^{-5}–10^{-4} hour^{-1} for m-cresol metabolism. Saturation kinetics were observed for amino acids and m-cresol. m-Cresol K_1 values for uptake often exceeded those for respiration by greater than a factor of ten. V_{max} values were low (amino acids, 10^{1}–10^{2} ng g^{-1} hour^{-1}; m-cresol, 10^{-1} ng g^{-1} hour^{-1}), whereas K_m values were quite high (amino acids, 10^{3}–10^{4} ng g^{-1}; m-cresol 10^{3}–10^{5} ng g^{-1}). Saturation was not observed in many horizons even at 10^{5} ng g^{-1} dry soil. Frequently, respiration obeyed saturation kinetics whereas uptake was first order. It is concluded that measuring only kinetics of respiration may lead to severe underestimations of biodegradation rates.

Introduction

The widespread occurrence of synthetic organic contamination in ground water is well documented [3, 11, 12, 14]. The most important mechanism for eliminating contaminants from these environments is microbial metabolism [1]. Recent studies have shown that subsurface microbes are present in numbers up to 10^{8} g^{-1} soil [18] and are capable of degrading various substrates [15, 19]. Some of these microbes and microbial communities have been characterized [5, 16, 20]. However, relatively little is known about rates of biodegradation by bacteria attached to soil particles and the environmental fates of metabolites. This information is needed to predict the persistence of pollutants in the subsurface environment.

Previously, kinetic parameters have been measured in nonhomogeneous environments by modeling rates of CO_2 evolution from mineralization of radiolabeled substrates [4, 6, 9]. These methods may underestimate biodegradation rates because they do not account for biological uptake or excretion of metabolic by-products. The objectives of this research effort were to develop and test methodology for measuring radiolabeled substrate metabolism, including nonrespiratory metabolism. The methodology was applied to a variety of soils, using radiolabeled amino acids and m-cresol as substrates.

Materials and Methods

Chemicals and Radioisotopes

Hydrogen peroxide (3% topical solution) was purchased from J. T. Baker Co. (Phillipsburg, New Jersey). Sodium azide, sodium pyrophosphate (PPi), and polyvinyl pyrrolidone (PVP) were obtained from Sigma Chemical Co. (St. Louis, Missouri). The compounds used as potential substrates for microbial metabolism were [ring-U-^{14}C] m-cresol (specific activity 54 mCi mmol^{-1}, Amersham Corp., Arlington Hts., Illinois), and a mixture of [U-^{14}C] amino acids (specific activity 0.1401 mCi mg^{-1}, New England Nuclear Corp., Boston, Massachusetts). [U-^{14}C] Toluene (10.9 mCi mmol^{-1}, Pathfinder Laboratories, St. Louis, Missouri) was used to determine substrate recovery after CO_2 collection. These compounds had radiochemical purities >97% as determined by the manufacturers. Ba$^{14}CO_3$ (specific activity 50 mCi mmol^{-1}, Amersham Corp., Arlington Hts., Illinois) working solutions were approximately 10^6 dpm 10 μl^{-1} in 1 M KOH. Scinti-Verse II (Fisher Scientific Corp., Fairlawn, New Jersey) scintillation cocktail was used for liquid scintillation counting.

Soils and Sampling

Subsurface soil samples were provided by Dr. J. T. Wilson (EPA, Robert S. Kerr Environmental Research Laboratory, Ada, Oklahoma) and were obtained from a pristine aquifer near Lula, Oklahoma. Many characteristics of the aquifer have been previously reported [17]. A general description of the soils is provided in Fig. 1. The aquifer was aerobic (2.6 mg liter^{-1} dissolved oxygen) which indicates a low biochemical oxygen demand. The water table was approximately 3.5 m below the surface. Intact cores from the various horizons were obtained aseptically using a soil corer and core-paring device described by Wilson et al. [20]. Samples were returned to the laboratory in an ice chest and stored at 5°C for up to 12 months. The samples used in this study contained 0.121, 0.167, 0.242, 0.220, and 0.148 ml pore water g^{-1} of soil for samples 9NN2, 9NN5, 9NN9, 9MM2 and 9LL7, and 9KK2, respectively. Pore volume was measured as the difference between the wet and dry weights per gram of stored soil samples. For some applications of these data, the calculation of concentrations and rates based on pore water concentrations may be useful. The pore water volumes reported above can be used to convert the units to mass volume^{-1} pore water.

Sample Preparation

Soil samples were homogenized by blending 700 ml sterile distilled water (d-H_2O) with 85 g soil in a blender that had been rinsed three times with 95% ethanol, then four times with sterile d-H_2O. While the blender was running, 10 ml aliquots of the resulting slurry were pipetted into 25 ml Pierce vials (Pierce Chemical Co., Rockford, Illinois) using an Oxford macropipettor (Brunswick Co., Inc., St. Louis, Missouri) with the tip cut off to enlarge the opening. Soil dry weight was

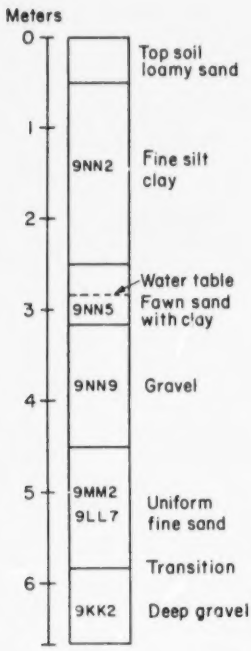

Fig. 1. Diagram of the Lula soil profile including the various horizons with their associated depths and descriptions. The water table fluctuates in the fawn sand layer.

determined by drying aliquots to constant weight at 105°C. Metabolically inhibited controls were prepared by adding NaN_3 (aq. 0.5% final concentration) to the samples.

Radiolabeled substrates were added to the vials using a micropipettor. The vials were completely filled with sterile d-H_2O and capped with (teflon-lined) Tufbond discs (Pierce Chemical Co.) and ring caps (Wheaton Scientific, Millville, New Jersey) such that air bubbles were excluded. The vials were inverted to prevent loss of substrates or CO_2 during the subsequent incubation. Samples were incubated at 17.0 ± 0.2°C (the in situ temperature). The time for sample preparation was typically 4 hours.

Four active samples and three inhibited controls were used at each of five substrate concentrations. In addition, three $Ba^{14}CO_3$-spiked samples without substrate were used to determine the CO_2 recovery efficiency.

CO_2 Recovery

To prepare for the recovery procedure, 150 μl of 1 M KOH was added to 40 ml Pierce vials, and the vials were topped with teflon-lined connectors (Wheaton Scientific). As depicted in Fig. 2A, the cap was removed from each sample and immediately replaced by one of the connector assemblies. The entire assembly was inverted, emptying the sample into the larger vial. The supernatant was used to rinse any remaining soil particles from the walls of the smaller vial. The connector and the smaller vial were then removed. Enough H_3PO_4 was added to the sample to bring the slurry to pH 2 (predetermined), and the vial was immediately capped with a CO_2 trap (Fig. 2B). The trap cup contained 150 μl of 1 N KOH.

The samples were shaken upright on a rotary shaker at 80 rpm for 20 hours to promote collection of CO_2 in the trap. The base was collected from the trap using a small fluted rectangle of Whatman #1 filter paper (Whatman Ltd., Maidville, England). The paper was placed in a scintillation vial

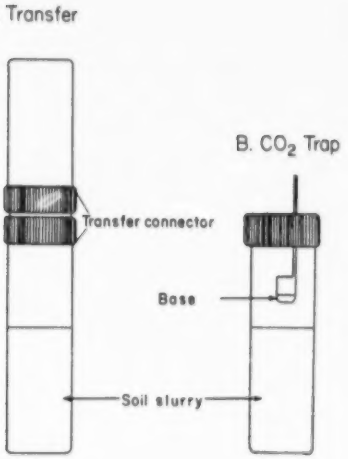

Fig. 2. Drawing of apparatus for $^{14}CO_2$ recovery. **(A)** The sample transfer assembly, including 25 and 40 ml Pierce vials joined by a teflon-lined connector, and **(B)** a 40 ml vial, containing a sample, capped with a basic CO_2 trap.

containing cocktail. All CO_2 samples and $Ba^{14}CO_3$ controls were counted 24 hours after collection to allow time for chemiluminescence to subside without significant loss of CO_2 from the samples.

Removal of Radioactivity from Soil Particles

The soil samples, after $^{14}CO_2$ recovery, were washed by adding 5 ml of a PVP and PPi solution (final concentrations 0.1% and 1%, respectively) directly to the 40 ml Pierce vials bringing the sample volumes to 30 ml. The samples were shaken on a rotary shaker at 200 rpm for 30 min then centrifuged at 120× *g* for 15 min. Five milliliters of the supernatant were filtered through 0.2 μm cellulose triacetate filters. The filters were washed with 5 ml of d-H_2O for amino acids experiments and 50% ethanol for m-cresol. The filter and a 1 ml aliquot of the first filtrate were placed in separate liquid scintillation vials for counting. The remainder of the supernatant was decanted and discarded.

The soil pellet was resuspended in 10 ml of 0.1% H_2O_2, and the previous procedures were repeated including the wash, centrifugation, filtration, and preparation of samples for counting. The remaining supernatant was decanted and discarded.

Quantitation of Soil-Associated Radioactivity

Ten milliliters of d-H_2O was added to the soil sample after the washing steps, and the sample was vortexed to uniformly distribute the soil. One milliliter of the suspension was quickly withdrawn and transferred with a micropipettor to a scintillation vial containing cocktail. (The tip had been previously cut from the pipette to create a larger opening.) Each sample was amended with 6 ml of water and shaken vigorously to form a gel.

AODC

Acridine orange direct counts were performed using a modification of the procedure described by Ghiorse and Balkwill [7]. Nuclepore filters (0.2 μm) (Nuclepore Corp., Pleasanton, California) were

prepared by staining them overnight with 2 g/liter irgalan black in 2% acetic acid. Fifty microliters of a 1:10 wt soil/wt water slurry were added to a filter on a tower with 10 ml prefiltered d-H_2O and 1 ml prefiltered 0.01% acridine orange stain. After approximately 3 min. vacuum was applied to the filter. The filter was removed from the tower and placed on a glass slide. One drop of type A immersion oil was placed on the filter and a coverslip was added. At least 20 microscope fields per filter were counted using epifluorescence microscopy.

Data Handling

All samples were counted for 10 min on a liquid scintillation counter. The counts were corrected to 100% efficiency using the channels ratio method.

Substrate turnover times (Tn) were calculated at each substrate concentration from the following equation, modified from Pfaender and Bartholomew [13]:

$$Tn = \frac{t}{f} = t\,\frac{A}{B + C + D}$$

where t is the incubation time in hours and f is the fraction of the added radiolabeled substrate metabolized. Specifically, A is the dpm added, B is the measured dpm in suspended particulates, C is the dpm respired, and D is the soil-associated dpm. A was obtained by counting three replicate aliquots of the substrate at the time of sample injection. B, C, and D are net values of biotic metabolism after subtraction of metabolically inhibited controls. Tn has units of hours and represents the time required for metabolism of a given concentration of the substrate. In these experiments t was constant so Tn was an inverse function of f.

Velocities (V) of metabolism were also calculated after the manner of Pfaender and Bartholomew [13] as follows:

$$v = S\,\frac{f}{t} = \frac{S}{t}\,\frac{B + C + D}{A}$$

where S is the substrate concentration ng g^{-1} dry soil; so velocities were expressed as ng substrate metabolized g^{-1} dry soil $hour^{-1}$ incubation.

A mass balance was calculated for each substrate concentration to determine the portion of added substrate recovered and the portion of recovered substrate accounted for by each of the metabolic fates.

Statistical Analysis

All analyses were performed with a microcomputer using the SYSTAT statistical package. V_{max} and K_m were calculated from saturation curves by nonlinear regression using the Michaelis-Menten equation as the model. The minimization technique used by SYSTAT was Quasi-Newton. The Simplex minimization technique was subsequently used to check the results (L. Wilkinson, SYSTAT: the System for Statistics, SYSTAT, Inc., Evanston, Illinois, 1986). Parameter starting values were obtained using the Haynes-Wolfe linear transformation of the Michaelis-Menten equation [10]. Convergence on local minima was avoided by repeating the estimations using various starting values.

First order rate constants (K_1) were estimated by performing linear regression on the first order portion of saturation curves. This was accomplished using the MGLH (Multivariate General Linear Hypothesis) module of SYSTAT.

Table 1. Effects of varying recovery time, acids, and headspace in the incubation vial on $^{14}CO_2$ recovery from $Ba^{14}CO_3$-spiked soil samples after a 24 hour incubation period

Experimental variable	Recovery (% of added dpm)	SD[a]
Recovery period (hours)[b,c]		
3	30	5
6	30	9
20	78	33
24	77	26
Acid and amount[b,d]		
H_3PO_4 (pH 2)	78	12
H_3PO_4 (2 X)	72	16
H_2SO_4 (pH 2)	59	7
Headspace[c,e]		
With headspace		
Base added	42	12
No base added	28	9
Without headspace		
Base added	27	5
No base added	23	7

[a] Standard deviation, 3 replicates
[b] Incubated with headspace
[c] Acidified to pH 2 with H_3PO_4
[d] 20 hour recovery period
[e] 6 hour recovery period

Results

CO_2 Recovery

Several experiments were performed to maximize the recovery of $^{14}CO_2$ using 24 hours incubated, $Ba^{14}CO_3$-spiked controls. The results of these experiments are provided in Table 1. The effect of varying the shaking time was studied. Samples that had been incubated with a headspace were acidified to pH 2 by injection of H_3PO_4 through the septum. The samples were placed on a rotary shaker and CO_2 was collected for various intervals. The data in Table 1 show that the mean CO_2 recovery was improved with up to 20 hours of collection and the maximum recovery obtained was 78%, with a high degree of variability. These data also show that recovery was not improved either by adding twice the volume of H_3PO_4, or by using H_2SO_4 in its place, although variability decreased using H_2SO_4.

In order to measure the biodegradation of volatile compounds it was desirable to incubate the samples without a headspace. Such an incubation necessitated transfer of the sample from the headspace-free vial to a larger vial for CO_2 recovery. The results of a test of the effects of headspace-free incubation on CO_2 recovery are given in Table 1. Samples incubated either with or without headspace were transferred to larger vials using connector caps (see Fig. 2). To

Table 2. Effect of PVP concentration on recovery of radiolabeled material on filters

PVP concentr. (%)	Metabolic[a] status	Activity on filters			
		dpm	SD[b]	% of added	SD
0.02	A	2,082	976	1.82	0.85
	I	1,259	36	1.10	0.03
0.10	A	3,237	1,101	2.82	0.95
	I	1,570	29	1.37	0.02
0.50	A	2,751	710	2.40	0.62
	I	1,350	123	1.18	0.10
1.00	A	3,001	1,140	2.62	0.99
	I	ND[c]	ND	ND	ND

Samples were incubated 12 hours with approximately 1.1×10^5 dpm of labeled amino acids. The PPi concentration was held constant at 1.0%

[a] Metabolic status: A = active, I = inhibited (NaN_3)

[b] Standard deviation

[c] Not determined

Table 3. Effect of wash time on recovery of radiolabeled material on filters

Wash time	Metabolic[a] status	Activity on filters			
		dpm	SD[b]	% of added	SD
10 min	A	1,951	303	1.70	0.26
	I	1,592	139	1.39	0.12
30 min	A	2,671	885	2.33	0.78
	I	1,445	68	1.26	0.05
1 hour	A	2,599	591	2.27	0.52
	I	1,550	220	1.35	0.19
3 hour	A	2,986	530	2.60	0.47
	I	1,406	69	1.23	0.07
6 hour	A	5,201	120	4.54	0.10
	I	1,709	52	1.49	0.05

Samples were incubated 12 hours with approximately 1.1×10^5 dpm of labeled amino acids

[a] Metabolic status. A = active, I = inhibited

[b] Standard deviation

half of the samples, 150 µl 1 M KOH was added before the transfer step to aid in retention of CO_2 by the samples during transfer.

Although incubation with headspace may have slightly improved recovery, headspace-free incubation was adequate. Adding KOH solution to headspace-free vials was difficult to do and improved recovery only slightly. Subsequently, samples were incubated headspace-free and transferred without addition of KOH. Each sample was acidified to pH 2 with H_3PO_4, and the CO_2 collection time was 20 hours.

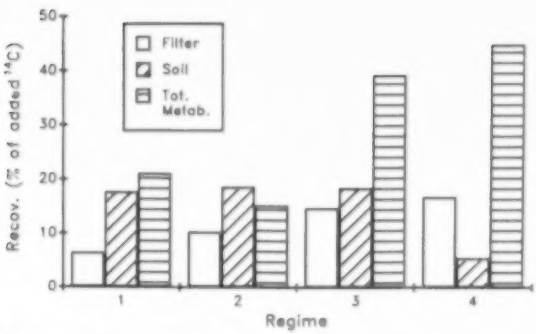

Fig. 3. Comparison of four soil-washing regimes by recovery in various fractions: (1) two washes with a PVP/PPi solution, (2) two washes with an H_2O_2 solution, (3) two washes with a PVP/PPi/H_2O_2 solution, and (4) one wash with PVP/PPi followed by one wash with H_2O_2. All effective concentrations of PVP, PPi, and H_2O_2 were 0.1, 1.0, and 0.1%, respectively.

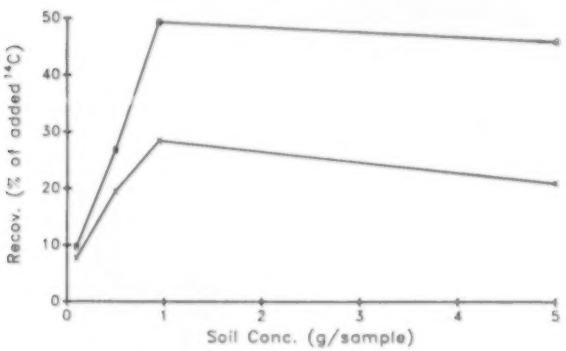

Fig. 4. Effects of soil concentration on amino acid respiration (×) and total metabolism (O). Uptake activity is the difference between the two.

Removal of Cells from Soil Particles

Experiments were performed to establish conditions for optimum recovery of labeled cells from soil particles. Soil samples were incubated overnight with known amounts of labeled amino acids, and the cells were recovered on filters as described. The concentration of polyvinyl pyrrolidone (PVP) in the wash solution was varied while holding the concentration of pyrophosphate (PPi) constant at 1.0%. The results are shown in Table 2. Increasing PVP concentration from 0.02 to 0.1% increased the recovery of labeled material on filters for both active and metabolically inhibited controls, but additional increases up to 1.0% did not result in increased recovery. Since PVP solutions become more viscous with increasing concentration, it is advantageous to use the lowest effective concentration to speed up filtration.

To determine the effect of various PVP/PPi washing times on label recovery

Table 4. Percent recovery of added ^{14}C amino acids and m-cresol in various sample fractions for all soils and all substrate concentrations combined. Medians with 25th and 75th percentiles are provided

Fraction	Metab.[a] status	Amino acids			m-Cresol		
		Median	25th	75th	Median	25th	75th
CO_2	A	11.25	5.45	16.57	1.38	0.68	3.44
	I	0.39	0.19	1.26	0.24	0.19	0.35
Soil and filter	A	25.11	13.94	38.37	21.67	16.08	27.22
	I	5.23	2.66	13.71	9.27	6.78	11.23
Total metabolism	Net	29.87	10.41	47.82	10.90	6.01	15.17
Total recovery	A	93.01	83.14	169.30	58.17	35.52	79.73
	I	106.03	84.42	169.18	54.15	25.60	77.27

[a] Metabolic status
A = active, I = inhibited (NaN_3), Net = A-I

from particles, samples were washed for up to 6 hours and filtered as previously described. As the data in Table 3 demonstrate, after 30 min no additional increase in recovery was seen until 6 hours. Two PVP/PPi washes (30 min each) were shown to be as effective in removing labeled material from particles as a single 14 hour wash (data not shown). Considering time and labor as well as recovery, it was decided to use two, 30 min washes.

The efficacies of four washing regimes for removing bound ^{14}C from the soil were compared. The results are shown in Fig. 3. The treatments included two PVP washes, two H_2O_2 washes, two washes with PVP and H_2O_2 combined, and a PVP wash followed by an H_2O_2 wash. The fourth treatment gave the most complete removal of soil-associated label. It also provided the highest recovery on filters and the highest measure of total metabolism. The adopted protocol was a PVP/PPi wash followed by a 0.1% H_2O_2 wash. AODC performed before and after the washes indicated that the cell recovery from soil particles was >90%.

Increasing the amount of soil in the test system up to 1 g resulted in a corresponding change in metabolic activity. Increasing the soil concentration above this level did not result in increased activity (Fig. 4). This could be the result of either mass transfer resistance or increased sorption of the substrate to the soil particles which decreases the apparent concentration. Samples in subsequent experiments contained between 0.5 and 1.0 g soil.

Mass Balances

Radioactivity was measured on the filters, in the filtrates, on the soil, and as trapped CO_2 to obtain a mass balance for the added label. Table 4 shows the median recoveries with 25th and 75th percentile values for labeled amino acids and m-cresol, combining all concentrations and all soil horizons. Since recovery varied as a function of substrate concentration, and lower concentrations were used most frequently, the distributions were skewed, making medians better

estimators of the distribution centers than means. Total metabolism was calculated by combining respiration and uptake [net (active − inactive) CO_2 production + net soil- and filter-associated activities].

For amino acids, median uptake activity was twice as high as respiration. Approximately 30% of the added label was metabolized over 1–3 day incubation periods. Total recoveries were near 100% for both active and inactive samples. Median CO_2 measurements in inhibited controls were <4% of the active measurements, whereas the corresponding value for uptake was 20%.

For m-cresol, net uptake was >10 times higher than net respiration. Approximately 10% of the originally added substrate was metabolized after 8–12 days of incubation, and about 55% was recovered. Control activities were higher for m-cresol than for amino acids. The higher uptake background (approximately 40% of active samples) could be the result of m-cresol binding to soil particles or microbial cells due to its more hydrophobic character. The higher respiration backgrounds (20% of active samples) are probably the result of volatilized m-cresol or its degradation products accumulating in the trap. Even with these somewhat higher inhibited controls, there were clear differences between active and inhibited samples.

Figure 5A and B shows the recovery of amino acids and m-cresol respectively, vs added substrate concentration. Results are shown for both active and inactive samples. For both amino acids and m-cresol, very high recoveries were consistently observed in filtrates at the lowest concentration tested. Several attempts to explain this phenomenon were unsuccessful. Near quantitative amino acid recoveries were observed throughout the rest of the concentration range. Except for the lowest concentrations, the recoveries of m-cresol varied between 25 and 75%. These less than quantitative recoveries were due to substrate volatilization during the filtrations, when the solution was exposed to the atmosphere.

Loss by volatilization was demonstrated by a study using toluene as the substrate, in which the point of substrate loss was determined. It had been shown that only 0.1% of the added toluene was accounted for by the mass balance at the highest concentration. In a separate study, after a 6 week incubation period, CO_2 was recovered as previously described. After the CO_2 recovery step the samples were not fractionated as in previous experiments. Instead, each sample was vortexed while three aliquots were removed and injected into vials for scintillation counting. CO_2 and the sample aliquots combined accounted for 103 ± 6% of the added radioactivity for both active and inhibited samples at all concentrations (range 0.1–100 $\mu g\ g^{-1}$ dry soil). This demonstrated that the loss of labeled material did not occur during incubation or CO_2 recovery, but during subsequent steps in the procedure, and therefore did not influence the concentration to which the microbes were exposed.

m-Cresol recoveries exhibited more variation than was seen with the amino acids. Examination of Fig. 5B reveals that the recovery of m-cresol in the gravel layers (9NN2 and 9KK2) was higher than the other recoveries. Since these were the layers highest in clay content, it is likely that greater adsorption resulted in a decreased substrate loss from the aqueous phase during the washing step. The recovery differences were not seen for amino acids (Fig. 5A), which do not adsorb as readily in this experimental system (data not shown).

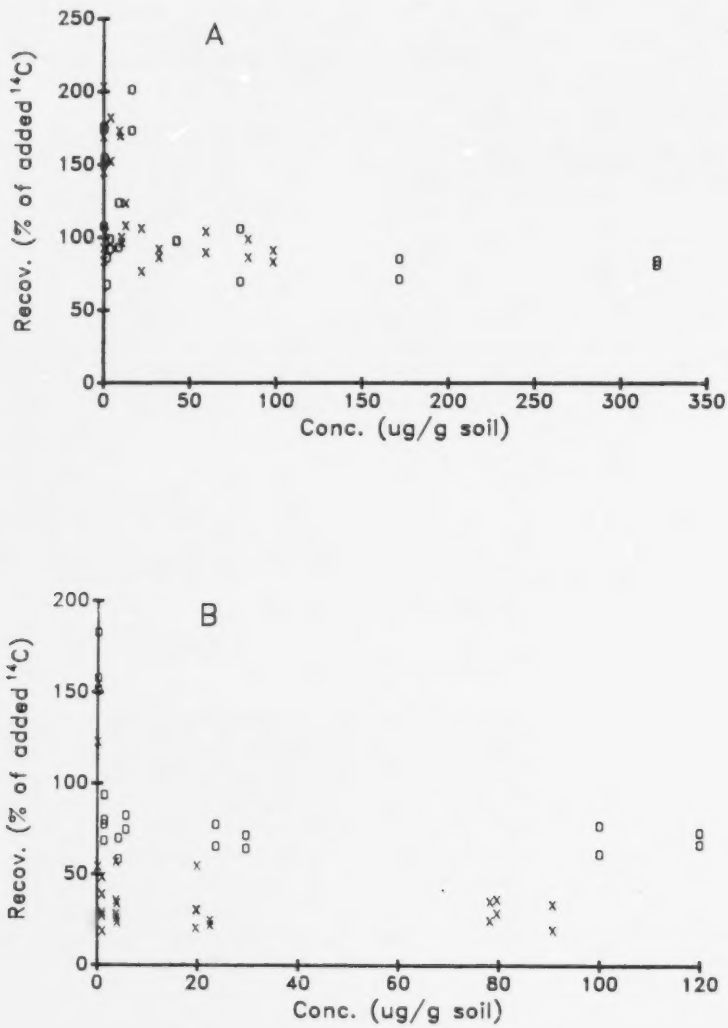

Fig. 5. ^{14}C-substrate recovery as a function of substrate concentration for (**A**) amino acids and (**B**) m-cresol. The data include both active and inactive samples from all soils. Gravel layers are represented by O and others are represented by ×.

Samples were stored at 5°C for various time periods up to 12 months. Though the effects of this storage on the microbial assemblages are not known, amino acid metabolism studies performed at various times during this period with the uniform fine sand produced substantially the same results (data not shown).

Table 5. First order rate constants for amino acid metabolism in the Lula soil profile, obtained by linear regression of velocity on substrate concentration in the linear portions of the kinetic curves

Soil[a]	Fraction	K_1 (hour^{-1})	SE[b]
9NN2	Respiration	4.86×10^{-3}	0.70×10^{-3}
	Uptake	4.53×10^{-3}	0.89×10^{-3}
	Total	8.69×10^{-3}	0.65×10^{-3}
9NN5	Respiration	2.59×10^{-3}	0.23×10^{-3}
	Uptake	4.33×10^{-3}	0.20×10^{-3}
	Total	6.92×10^{-3}	0.42×10^{-3}
9NN9	Respiration	1.02×10^{-2}	0.03×10^{-2}
	Uptake	9.86×10^{-3}	0.20×10^{-3}
	Total	2.00×10^{-2}	0.04×10^{-2}
9MM2	Respiration	1.13×10^{-2}	0.04×10^{-2}
	Uptake	2.57×10^{-3}	0.31×10^{-3}
	Total	1.98×10^{-2}	0.09×10^{-2}
9KK2	Respiration	2.65×10^{-5}	1.25×10^{-5}
	Uptake	5.21×10^{-4}	0.86×10^{-4}
	Total	5.44×10^{-4}	0.96×10^{-4}

[a] Refer to Fig. 1 for description of the soil profile and sample location
[b] Standard error of the estimate

Kinetics of Biodegradation

The first order rate constants (K_1) for amino acid metabolism are provided in Table 5. The rate constants of uptake and respiration were similar in any given horizon. This indicates that neither process dominates the microbial metabolism of amino acids. It also suggests that both processes are important to the survival of organisms in this environment. The 9NN9 and 9MM2 samples from the saturated zone demonstrated the highest rate constants of total metabolism. The lowest rates were observed in the adjacent deep gravel layer (9KK2). In general, rates of amino acid metabolism were in the high 10^{-3} to low 10^{-2} hour^{-1} range. Standard errors were, on the average, 11% of the estimates, indicating that the data fit the first order model reasonably well.

For m-cresol (Table 6), uptake and respiration values were similar in each of the upper two horizons (unsaturated zone). Uptake exceeded respiration by approximately a factor of 10 in the 9NN9 and 9KK2 horizons and by a factor of 50 in the 9LL7 horizon. The uptake rate constants were lower than those for amino acids, and the respiration constants were lower yet. This does not de-emphasize the importance of respiratory metabolism, but rather emphasizes the relative recalcitrance of m-cresol. K_1 values for respiration were usually in the range 10^{-5} hour^{-1}, and uptake values were on the order of 10^{-4} hour^{-1}. Standard errors were typically 8% of the estimated values, indicating that these data also fit the first order kinetic model.

Table 7 contains the results of nonlinear curve-fitting for amino acid metabolism using the NONLIN program in SYSTAT (Evanston, Illinois). Saturation was observed for both respiration and uptake in the fawn sand (9NN5)

Table 6. First order rate constants for m-cresol metabolism in the Lula soil profile, obtained by linear regression of velocity on substrate concentration in the linear portions of the kinetic curves

Soil[a]	Fraction	K_1 (hour^{-1})	SE[b]
9NN2	Respiration	6.62×10^{-4}	0.71×10^{-4}
	Uptake	6.31×10^{-4}	0.50×10^{-4}
	Total	1.03×10^{-3}	0.08×10^{-3}
9NN5	Respiration	9.12×10^{-5}	2.01×10^{-5}
	Uptake	7.55×10^{-5}	0.50×10^{-5}
	Total	1.45×10^{-4}	0.14×10^{-4}
9NN9	Respiration	1.57×10^{-5}[c]	0.12×10^{-5}
	Uptake	2.91×10^{-4}	0.04×10^{-4}
	Total	3.06×10^{-4}	0.04×10^{-4}
9LL7	Respiration	1.04×10^{-5}[c]	0.06×10^{-5}
	Uptake	5.02×10^{-4}	0.28×10^{-4}
	Total	5.07×10^{-4}	0.28×10^{-4}
9KK2	Respiration	2.64×10^{-5}	0.30×10^{-5}
	Uptake	4.80×10^{-4}	0.27×10^{-4}
	Total	4.87×10^{-4}	0.21×10^{-4}

[a] Refer to Fig. 1 for a description of the soil profile and location of the samples
[b] Standard error of the estimate
[c] Uptake value significantly greater than respiration (t-test, 5 comparisons, overall $\alpha = 0.001$); none of the other differences between uptake and respiration were significant

Table 7. Kinetic parameters of amino acid metabolism in the Lula soil profile

Soil[a]	Sample fraction	V_{max} (ng/hour/g)	SE[b]	K_m (ng/g)	SE[b]
9NN2	Respir.	NS[c]		NS	
	Uptake	NS		NS	
	Total	NS		NS	
9NN5	Respir.	6.73×10^{1}	1.23×10^{1}	1.48×10^{4}	0.76×10^{4}
	Uptake	1.14×10^{2}	0.14×10^{2}	1.39×10^{4}	0.41×10^{4}
	Total	1.82×10^{2}	0.28×10^{2}	1.49×10^{4}	0.63×10^{4}
9NN9	Respir.	NS		NS	
	Uptake	NS		NS	
	Total	NS		NS	
9MM2	Respir.	1.25×10^{2}	0.52×10^{2}	1.77×10^{4}	0.79×10^{4}
	Uptake	5.48×10^{1}	2.80×10^{1}	3.55×10^{3}	1.97×10^{3}
	Total	1.97×10^{3}	0.27×10^{2}	9.10×10^{3}	1.34×10^{3}
9KK2	Respir.	1.35×10^{1}	0.16×10^{1}	2.03×10^{3}	1.80×10^{3}
	Uptake	NS		NS	
	Total	NS		NS	

V_{max} and K_m were calculated using the Michaelis-Menten model
[a] Refer to Fig. 1 for a description of the soil profile and sample locations
[b] Standard error of the estimate
[c] Not saturated

Table 8. Kinetic parameters of m-cresol metabolism in the Lula soil profile

Soil[a]	Sample fraction	V_{max} (ng/hour/g)	SE[b]	K_m (ng/g)	SE[b]
9NN2	Respir.	1.72×10^{1}	0.09×10^{1}	2.65×10^{4}	0.36×10^{4}
	Uptake	NS[c]		NS	
	Total	NS		NS	
9NN5	Respir.	5.92×10^{-1}	0.49×10^{-1}	1.29×10^{3}	0.56×10^{3}
	Uptake	NS		NS	
	Total	NS		NS	
9NN9	Respir.	NS		NS	
	Uptake	NS		NS	
	Total	NS		NS	
9LL7	Respir.	3.86×10^{0}	0.18×10^{0}	2.70×10^{5}	0.12×10^{5}
	Uptake	NS		NS	
	Total	NS		NS	
9KK2	Respir.	7.67×10^{-1}	0.91×10^{-1}	1.97×10^{4}	0.81×10^{4}
	Uptake	NS		NS	
	Total	NS		NS	

V_{max} and K_m were calculated using the Michaelis-Menten model
[a] Refer to Fig. 1 for a description of the soil profile and sample locations
[b] Standard error of the estimate
[c] Not saturated

and uniform sand (9MM2) horizons, but only for respiration in the confining gravel layer (9KK2). The fine silt in the unsaturated zone (9NN2) and the intermediate gravel (9NN9) demonstrated first order kinetics throughout the tested range of substrate concentrations. V_{max} values were on the order of 10^{1}–10^{2} ng hour^{-1} g^{-1} soil and the highest value was only four times the lowest. Similarly, the range of observed K_m values was contained within a factor of nine.

For m-cresol (Table 8), uptake followed first order kinetics exclusively whereas respiration exhibited saturation kinetics. The associated K_m values were comparable to values for amino acid respiration. In contrast, V_{max} values of m-cresol respiration were typically one to two orders of magnitude lower than for amino acids. The failure of some of the horizons to exhibit saturation kinetics is presumed to be the result of extremely high K_m values. Such values have been previously noted [4].

No apparent relationship was seen between kinetic parameters and depth over the entire profile. However, V_{max} and K_m did decrease from the surface to the water table (9NN2 to 9NN5) and from the water table to the confining layer (9MM2 and 9LL7 to 9KK2). This is consistent with previous observations of biodegradation rates in subsurface profiles [19]. Since kinetic data from other studies are scanty, generalized relationships between parameters and depth cannot be firmly established.

Discussion

Soon after the advent of the heterotrophic uptake technique [21] it was recognized that both mineralization and cellular uptake measurements were need-

ed to adequately quantify substrate turnover rates [8]. From 1971 until the present, however, biodegradation in nonhomogeneous environments has commonly been reported as respiration activity alone, because methods for measuring cellular uptake of radiolabeled substrates were not available [4, 6, 9]. Because most cells in soil and sediments are associated with particles, as is any adsorbed label, it is often difficult to distinguish between substrate incorporated into cells associated with soil particles and that sorbed. This is especially difficult for pollutants which are often slowly metabolized and highly sorbed where there would be low biological activity against a large abiotic background.

The development of the methodology described in this report is due, in part, to the availability of techniques for effectively removing cells from particulate matter [2]. With the cells separated from the soil it is relatively simple to isolate the two and determine the radioactivity in the cell fraction. Using these techniques it is possible to observe the kinetics of substrate respiration and uptake by soil-associated microbes. As defined here, uptake may include (1) substrate which is preferentially and irreversibly sorbed to actively metabolizing microbes (radioactivity in inactive controls was subtracted, and the cells were washed on the filters with water or 50% ethanol to minimize this factor), (2) excreted metabolic by-products, and (3) assimilated substrate.

To estimate the proportion of metabolized substrate that was excreted, sorption was assumed negligible. The numbers of cells removed from the soil by PVP/PPi washing (free) and those remaining on the soil (bound) were determined by AODC. The specific activity of the free cells was calculated from the filter-associated activity and AODC (dpm $cell^{-1}$). The uptake activity of the bound cells was assumed to be equal to that of the free cells, because they were all originally on the soil. The expected soil-associated dpm due to bound cells was then calculated. This value represents the amount of substrate taken up by the cells on the soil. When this amount of radioactivity is subtracted from the amount measured on the soil, the difference should represent noncellular biological uptake.

Using this approach, it was estimated that 15–25% of the metabolized substrate was converted to soil-associated, noncellular, biological material, independent of variations in substrate and concentration. The estimate does not account for noncellular material that was removed from the soil by PVP washes.

In general, the kinetics of CO_2 production observed here support previous findings for subsurface environments. The rates of metabolism were low [19], the degradative potentials (V_{max}) were low, and the half-saturation constants were high [4]. The observation of high K_m implies that V_{max} is not as useful as K_1 for predicting biodegradation rates in this environment. This is because the substrate concentration required to reach V_{max} will not usually be available. It is clear from these results, however, that measuring CO_2 evolution alone can lead to severe underestimates of biodegradation; assimilatory metabolism apparently accounts for a large proportion of total metabolism and may, on occasion, be the dominant metabolic process. Also, the kinetics of the two processes are not necessarily similar (i.e., respiration may obey Michaelis-Menten kinetics whereas uptake kinetics are first order).

Relatively long turnover times were observed at the low concentrations (not presented) which strongly indicated concentration thresholds below which metabolic rates decreased sharply. It was not clear, however, whether the thresholds

were due to decreases in biological responses or to some extraneous factor (e.g., substrate availability or isotope dilution).

The toluene recovery experiment was designed to test whether very volatile compounds can be employed as substrates with no threat of significant substrate losses. It is evident from that experiment that low recoveries are not the result of CO_2 losses. It is strongly suspected that the losses were due to toluene volatilization in the filtration apparatus under vacuum. Such losses would have no effect on measured rates of metabolism. This suggestion is further supported by the kinetic data.

Under most circumstances, the proper model for regression would be the nonweighted equation of Li [10]. However, due to decreasing variance in the Y variable with increasing substrate concentration, the data were weighted using the Michaelis-Menten equation. In general, the two models resulted in the same parameter estimates, but the model of Li [10] occasionally led to nonconvergence due to the large variances in the low concentration range. The unweighted data were rates of metabolism (f/t) whereas the transformed data were the familiar Michaelis-Menten velocities (fs/t) which are equally, if not more, biologically meaningful. Here, f is the fraction of substrate with concentration S metabolized in time equal to t.

In this study, soil slurries were used for making metabolism measurements, which resulted in a dilution of the biomass relative to that in the aquifer. Since the addition of similar amounts of substrate to a nondiluted aquifer sample would result in a higher concentration of both substrate and organisms, the rates reported may be underestimates of what would be observed in nature. If biodegradation followed first order kinetics, then the amount of substrate metabolized would vary linearly with the dilution of the sample due to concentration changes, but the rate constant would remain the same. If degradation were second order, then diluting the soil sample would dilute the organisms and would produce a lower rate constant. Since the soil samples were diluted approximately 110-fold in this study, apparent first order rate constants, given aquifer solids concentrations, could be obtained by multiplying the observed constants by the dilution factor. However, making this extrapolation assumes that mass transport and sorption are not significant influences on biodegradation.

Data presented here (Fig. 4) and elsewhere [4] show that there is an increase in metabolism as greater amounts of soil are added to a constant amount of substrate, but the increase is not proportional to the amount of solids added. It appears likely that two competing processes may be involved. As the solids concentration increases, the amount of active biomass increases proportionally, which should result in a correspondingly faster rate of metabolism. However, at higher solids concentrations, there is also more sorptive surface relative to the volume; so a greater proportion of the substrate may be sorbed and therefore unavailable to the organisms. The effect is a decrease in the effective substrate concentration and lower rates of metabolism. At high solids concentrations, mass transport limitations may also produce lower rates.

It seems clear that adjusting rates to aquifer solids concentrations using the second order extrapolation would overestimate the actual rate. More research on the relationships between sorption and biodegradation are needed before measured laboratory rates can be reasonably extrapolated to field conditions.

Further research is also needed to determine the environmental factors—physical, chemical, and microbiological—that control metabolic rates in subsurface soils. One topic of particular interest is microbial adaptation under varying conditions, both as it occurs in nature and in controlled laboratory experiments.

Acknowledgments. The authors wish to thank Dr. John T. Wilson for help in the collection of subsurface soil samples. This work was supported by Cooperative Agreement CR-811828 with the Environmental Protection Agency.

References

1. Alexander M (1981) Biodegradation of chemicals of environmental concern. Science 211: 132–138
2. Balkwill DL, Ghiorse WC (1985) Characterization of subsurface bacteria associated with two shallow aquifers in Oklahoma. Appl Environ Microbiol 50:580–588
3. Bitton G, Gerba CP (1984) Groundwater pollution microbiology: the emerging issue. In: Bitton G, Gerba CP (eds) Groundwater pollution microbiology. Wiley, New York, pp 1–7
4. Dobbins DC, Thornton-Manning JR, Jones DD, Federle TW (1987) Mineralization potential for phenol in subsurface soils. J Environ Qual 16:54–58
5. Federle TW, Dobbins DC, Thornton JR, Jones DD (1986) Microbial biomass, activity, and community structure in subsurface soils. Ground Water 24:365–374
6. Ferroni GD, Leduc LG, Winterhalder EK (1985) The measurement of mineralization activity in soils. Soil Biol Biochem 17:727–728
7. Ghiorse WC, Balkwill DL (1983) Enumeration and morphological characterization of bacteria indigenous to subsurface environments. Dev Indust Microbiol 24:213–224
8. Hamilton RD, Austin KE (1967) Assay of relative heterotrophic potential in the sea: the use of specifically labeled glucose. Can J Microbiol 13:1165–1173
9. Harrison MJ, Wright RT, Morita RY (1971) Method for measuring mineralization in lake sediments. Appl Microbiol 21:698–702
10. Li WKW (1983) Consideration of errors in estimating kinetic parameters based on Michaelis-Menten formalism in microbial ecology. Limnol Oceanogr 28:185–190
11. McCarty PL, Reinhard M, Rittman BE (1981) Trace organics in groundwater. Environ Sci Technol 15:40–48
12. Page GW (1981) Comparison of groundwater and surface water for patterns and levels of contamination by toxic substances. Environ Sci Technol 15:1475–1481
13. Pfaender FK, Bartholomew GW (1982) Measurement of aquatic degradation rates by determining heterotrophic uptake of radiolabeled pollutants. Appl Environ Microbiol 44:159–164
14. Pye VI, Patrick R (1983) Groundwater contamination in the United States. Science 221: 713–718
15. Ventullo RM, Larson RJ (1985) Metabolic diversity and activity of heterotrophic bacteria in ground water. Environ Toxicol Chem 4:759–771
16. White DC, Smith GA, Gehron MJ, Parker JH, Findlay RH, Martz RF, Fredrickson HL (1983) The groundwater aquifer microbiota: biomass, community structure, and nutritional status. Dev Ind Microbiol 24:201–211
17. Wilson JT, Miller GD, Ghiorse WC, Leach FR (1986) Relationship between the ATP content of subsurface material and the rate of biodegradation of alkyl benzenes and chlorobenzene. J Contam Hydrol 1:163–170
18. Wilson JT, McNabb JF (1983) Biological transformation of organic pollutants in groundwater. EOS 64:505–507
19. Wilson JT, McNabb JF, Balkwill DL, Ghiorse WC (1983) Enumeration and characterization of bacteria indigenous to a shallow watertable aquifer. Ground Water 21:134–142
20. Wilson JT, McNabb JF, Wilson BH, Noanan MJ (1983) Biotransformation of selected organic pollutants in groundwater. Dev Ind Microbiol 24:225–233
21. Wright RT, Hobbie JE (1965) The uptake of organic solutes in lake water. Limnol Oceanogr 9:163–178

NOTICE

This document has been reviewed in accordance with U.S. Environmental Protection Agency policy and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

This article documents research that was conducted under the auspices of the U.S. Environmental Protection Agency (USEPA). As an arm of the government, the USEPA has a paid up, non-exclusive, irrevocable, worldwide license to reproduce, prepare derivative works, and distribute to the public all of the material contained herein, and to grant others acting on its behalf the right to do so. The USEPA grants to the National Technical Information Service, Department of Commerce, the right to act as its agent in the distribution of this material.